# The Effect of Nisin and Monensin on Ruminal Fermentations In Vitro

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## Introduction

In ruminant animals, feedstuffs are fermented in the rumen, and this method of digestion promotes fiber and non-protein nitrogen utilization. Animals can utilize ruminal volatile fatty acids and microbial protein, but methane, heat and ammonia are also produced. When methane production is the primary mechanism of reducing equivalent disposal, the ratio of acetate to propionate increases and energy retention by the animal declines. Since the 1970's, nutritionists have sought chemical additives to decrease fermentation losses and increase the useful end-products of ruminal fermentation.

Chlorinated hydrocarbons are potent inhibitors of methane production in vitro, but these additives are inactivated in vivo . Ionophores were developed as coccidiostats, but they can alter ruminal fermentation by dissipating the ion gradients of gram-positive bacteria. Monensin, the most commonly used ionophore, was originally marketed as a methane inhibitor and propionate enhancer, but it also appears to decrease wasteful amino acid degradation and ammonia accumulation. Monensin often decreases food intake. This latter effect is often explained by increases in energy availability, but a learned aversion to monensin has been demonstrated.

Animals absorb monensin, but much is returned to the gut in bile via the enterohepatic circulation. The $LD_{50}$ of monensin in ruminants is 20 to 40 times the recommended dose, but horses have an $LD_{50}$ that is approximately 10-fold lower than cattle. Doses of monensin that are commonly used for cattle will kill horses, and this finding has created serious problems for the feed industry. Humans exposed to monensin during its manufacture have reported symptoms that included headache, nausea, nosebleed and skin rash, and people feeding monensin to animals have complained of headache and dizziness.

Many lactic acid bacteria produce small peptides that increase the transmembrane flux of protons, and these bacteriocins are primarily effective against gram-positive bacteria. Nisin was the first recognized bacteriocin, and it is produced by Lactococcus lactis, a common starter culture for cheese making. Nisin is not toxic to animals, and it has been approved by the FDA for use in human foods. Nisin has been used to control food-borne pathogens, but its effects on ruminal fermentation have not been examined.

Based on its ability to inhibit gram-positive ruminal bacteria, it appeared that nisin might be a useful additive for controlling or modifying ruminal fermentations. The following experiments compared effects of monensin and nisin on mixed and pure cultures of ruminal bacteria in vitro.

## Materials and Methods

Mixed ruminal bacteria were incubated anaerobically in tubes (10 ml of incubation fluid) containing 0.1 g of ground alfalfa hay. Tubes were sealed with butyl rubber stoppers and aluminum crimps and incubated at 39°C. Nisin and monensin were prepared as separate anaerobic solutions dissolved in 95% ethanol. Nisin solutions were covered in aluminum foil to prevent degradation by light. All inhibitors were stored at 4°C. Nisin and monensin were added to each culture tube to achieve final concentrations needed and equal volumes of ethanol were added as controls. The ethanol concentration was never greater than 1.6% (vol/vol). A gas sample (0.5 ml) was removed from each tube after 24 h of incubation and analyzed for methane and hydrogen. The cell-free supernatant was stored at -15°C. Mixed ruminal bacteria were added anaerobically to tubes (10 ml of 1/3 ruminal fluid) containing 20 mg of commercial starch, Trypticase, or ball-milled cellulose. The tubes were incubated at 39°C in a water bath for 24 h. The cell-free supernatants were prepared and stored as described above. To see if the ruminal bacteria could adapt to monensin or nisin, the mixed ruminal bacteria were transferred successively (4 times) in fresh tubes that contained 1/3 ruminal fluid (clarified by centrifugation and autoclaved), 2/3 basal medium (see above), 20

mg starch, Trypticase, or 9 d ball-milled cellulose and either 5 mM monensin or nisin. Cultures of ruminal obligate amino acid-fermenting bacteria were grown anaerobically on Trypticase or Casaminoacids with different concentrations of nisin and monensin (as above). To study adaptation, the obligate amino acid-fermenting bacteria were transferred successively (4 times) with the highest dose of either monensin or nisin that did not inhibit growth. Fermentation acids were measured by HPLC. Ammonia was assayed by a colorimetric method.

**Results**

When mixed ruminal bacteria and alfalfa were incubated in vitro, monensin and nisin both inhibited methane production so long as the concentrations were greater than 1 mM. Monensin- and nisin-dependent methane depressions caused a decrease in the acetate to propionate ratio (4.5 to 3.0). Total volatile fatty acid production was decreased by both monensin and nisin addition at concentrations greater than 2 mM. Starch-digesting ruminal bacteria were initially inhibited by monensin and nisin, but this effect disappeared after 2 to 4 transfers. Nisin always inhibited cellulolytic bacteria, but the nisin-dependent inhibition of cellulose digestion was no greater than the inhibition caused by monensin. Monensin and nisin also inhibited amino acid degradation, and nisin was more effective than monensin in controlling the growth of Clostridium aminophilum, an obligate amino acid-fermenting ruminal bacterium that can tolerate low concentrations of monensin. Because nisin was as potent as monensin, bacteriocins such as nisin may have potential as feed additives.

**Discussion**

Monensin and nisin have very different chemistries, but both of these molecules are able to translocate ions across cell membranes. Monensin is a donut-shaped ionophore that complexes monovalent metal ions and allows them to pass across the cell membrane. Once the metal ion has been released, the carboxyl group then binds a proton and shuttles it in the opposite direction. Nisin is a short peptide that aggregates to form a pore through the cell membrane. Nisin dissipates both components of the protonmotive force, and nisin appears to produce nonselective channels for ions, amino acids and ATP.

Ionophore sensitivity is closely correlated with cell wall structure. Gram-negative bacteria have an outer membrane that keeps monensin from reaching the cell membrane, but gram-positive bacteria lack this defense mechanism and are generally more sensitive. The outer membrane also serves as a barrier to nisin, but recent work indicated that nisin was able to increase the oxygen consumption of *Escherichia coli*, a gram-negative bacterium. Ruminal bacteria produce a variety of peptidases and proteinases, but nisin is an unusual peptide. The sulfur linked alanine residues (lanthionine) of nisin are resistant to common proteinases.

Because monensin is typically fed at a daily dose of 350 mg per day and the ruminal volume of mature cattle is approximately 70 liters, the in vivo concentration of monensin would be approximately 5 ppm or 7.2 mM. These calculations, however, do not consider the fact the bacterial concentration in vivo is very high and the observation that monensin can bind to gram-negative bacteria as well as feed particles. Because the effective in vivo dose of monensin is less than 5 mM, we decided to examine the effect of monensin and nisin in a dose-dependent fashion.

Monensin has little direct effect on methanogenic bacteria, but it inhibits ruminal bacteria that produce hydrogen, a precursor of ruminal methane. Because ruminal methane can also be derived from formate, monensin never causes a complete inhibition of methane production. Our in vitro experiments indicated that monensin and nisin decreased methane production 47 and 32%, respectively. Monensin and nisin both decreased the ratio of acetate to propionate production and this result is consistent with their ability to decrease methane production and divert reducing equivalents to other disposal mechanisms (e.g., propionate).

When mixed ruminal bacteria were incubated in vitro with alfalfa hay, monensin and nisin did not cause an inhibition of volatile fatty acid production until the

concentration was greater than 2 mM, and the maximum inhibition was 18%. Purified substrates were more sensitive to monensin and nisin, and even low doses (≤ 1 mM) caused a significant inhibition. Starch was less sensitive than cellulose, but even starch-fermenting bacteria were inhibited by low concentrations of monensin or nisin. Monensin and nisin-dependent inhibitions of starch fermentation disappeared in subsequent transfers, but the cellulolytic bacteria never seemed to adapt.

Ruminal amino acids can be deaminated by carbohydrate-fermenting ruminal bacteria, but these bacteria have low specific activities of ammonia production and are in most cases monensin resistant. The rumen also has a small but highly active population of monensin-sensitive, obligate amino acid-fermenting bacteria. When cattle were supplemented with monensin, the specific activity of ammonia production and steady state concentration of ammonia decreased 30 to 50%, and there was a 10-fold decline in the numbers of obligate amino acid-fermenting bacteria. Monensin and nisin were both able to decrease Trypticase deamination, and little, if any, adaptation was observed.

Obligate amino acid-fermenting ruminal bacteria are sensitive to monensin in vitro, but *C. aminophilum* is more resistant to monensin than *C. sticklandii* or *P. anaerobius* . When animals are fed monensin, the amount of rRNA that would hybridize *with C. sticklandii* or *P. anaerobius* probes declined to undetectable amounts, but *C. aminophilum* persisted. The present work indicates that *C. aminophilum* was 8-fold more resistant to nisin than monensin and adaptation was not observed.

**Conclusion**

Nisin and monensin are both able to inhibit ruminal methane, decrease acetate to propionate ratios and prevent amino acid deamination. Nisin and monensin have similar effects on carbohydrate fermentation, but nisin is a more potent inhibitor of obligate amino acid-fermenting ruminal bacteria. There was little evidence that nisin was inactivated by ruminal bacteria, but in vivo feeding trials are obviously needed to evaluate more fully the use of bacteriocins as ruminant feed additives.